

CHAPTER II

LITERATURE REVIEW

II.1. ETHANOL INDUCED HEPATIC TISSUE CHANGES

II.1.1. Ethanol

Ethanol is a commonly used substance among people all over the world with its intoxicating effects and potential for abuse. The main component of all alcoholic drinks is ethanol (ethyl alcohol). Ethanol is one of direct hepatotoxins agents, ethanol ingested in high doses impairs tissues by a variety of mechanisms,¹⁷ oxidative stress plays surely a crucial role. Most ethanol is broken down (i.e., metabolized) in the liver through a series of chemical reactions, known as oxidation reactions, which involve hydrogen and oxygen atoms. In the predominant biological pathway for ethanol metabolism, called the alcohol dehydrogenase pathway (ADH), a second pathway of ethanol metabolism, the microsomal ethanol-oxidizing system (MEOS), is activated by long-term heavy ethanol consumption.¹⁸

II.1.2. Definition of liver injury induced by ethanol

Hepatic tissue changes related to ethanol consumption, that varies from simple steatosis to cirrhosis. Often, the continuing heavy ethanol consumption that progress from fatty liver to hepatitis to cirrhosis. These are not necessarily distinct stages of evolution of disease, but rather, multiple stages that may be present simultaneously in a given individual. These are often grouped into three histological stages of ALD: fatty liver or simple steatosis, alcoholic hepatitis, and

chronic hepatitis with hepatic fibrosis or cirrhosis. These latter stages may also be associated with a number of histologic changes (which have varying degrees of specificity for ALD), including the presence of Mallory bodies.¹⁹

II.1.3. Ethanol metabolism

Most tissues of the body, including the skeletal muscles, contain the necessary enzymes for oxidative or nonoxidative metabolism of ethanol. However, the major site of ethanol metabolism is the liver. Within the liver, there are three enzyme systems for metabolism of ethanol, These enzymes are: - Cytosolic alcohol dehydrogenase (ADH) uses nicotinamide adenine dinucleotide (NAD) as an oxidizing agent.

1. The microsomal ethanol-oxidizing system (MEOS) uses nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen. The central enzyme of MEOS is cytochrome P-450 2E1 (CYP2E1). Ethanol up regulates CYP2E1, and the proportion of alcohol metabolized via this pathway increases with the severity and duration of alcohol use.
2. Peroxisomal catalase uses hydrogen peroxide as an oxidizing agent.²⁰

II.1.4. Factors that influence the liver injury

II.1.4.1. Genetic factors

Although the evidence to prove a genetic predilection to alcoholism is adequate, the role of genetic factors in determining susceptibility to alcoholic liver injury is much less clear. The fact remains that only a small fraction of even heavy alcoholics develop severe liver disease (ie, cirrhosis). The studies have yielded

inconsistent results, as have studies of genetic polymorphisms of collagen, ADH, ALDH, and CYP2E1. The genetic factor that most clearly affects susceptibility is sex. For a given level of ethanol intake, women are more susceptible than men to developing alcoholic liver disease.²¹

II.1.4.2. Malnutrition

In the past, nutritional deficiencies were assumed to play a major role in the development of liver injury. This view changed after key studies, that demonstrated that alcohol ingestion could lead to steatohepatitis and cirrhosis in the presence of a nutritionally complete diet. However, subsequent studies have suggested that enteral or parenteral nutritional supplementation in patients with alcoholic hepatitis may improve survival.²²

II.1.5. Diagnosis of liver injury induced by ethanol

II.1.5.1. Clinical symptoms

Fatty liver typically either is asymptomatic or present with nonspecific symptoms that do not suggest acute liver disease. Supporting features on physical examination include an enlarged and smoothly, but rarely tender, liver. While Alcoholic hepatitis caused by ethanol is a syndrome with a spectrum of severity, and therefore manifesting symptoms vary. Whereas clinical symptoms of alcoholic hepatitis caused by ethanol may be more severe and specific symptoms, that include hepatomegaly, jaundice, ascites, spider angiomas, fever, and encephalopathy.^{19, 23}

II.1.5.2. Laboratory diagnosis of liver injury induced by ethanol

II.1.5.2.a. Laboratory tests

Laboratory tests show increases in serum aspartate aminotransferase (AST), Gamma glutamyl transferase (GGT), while the increase in alanine aminotransferase (ALT) is less pronounced. Other markers include hypoalbuminemia, prolonged prothrombin time (PT), increase in mean corpuscular volume (MCV) of erythrocytes.²⁴

II.1.5.2.b. Liver biopsy

Although not essential in the management of ALD, a liver biopsy is useful in establishing the diagnosis. The 2010 American Association for the Study of Liver Diseases (AASLD) alcoholic liver disease (ALD) practice guideline recommends considering liver biopsy for patients whose diagnosis is reasonably uncertain and for patients likely to undergo medical treatment for severe alcoholic hepatitis, and liver biopsy is useful in establishing the stage and severity of liver disease.²⁵ Typical histologic findings of fatty liver include fat accumulation in hepatocytes that is often macrovesicular, but it is occasionally microvesicular. Whereas the classic histologic features of alcoholic hepatitis include inflammation and necrosis, which are most prominent in the centrilobular region of the hepatic acinus. The inflammatory cells infiltrate, located primarily in the sinusoids, consists of polymorphonuclear cells and mononuclear cells. In addition to inflammation, Some hepatocytes contain fat droplets (steatosis, green arrow), whereas others may contain intracellular, amorphous, eosinophilic inclusion

bodies called Mallory bodies (red arrow), which are often surrounded by neutrophils (yellow arrow) (hematoxylin and eosin).^{19,26} (Figure 1).

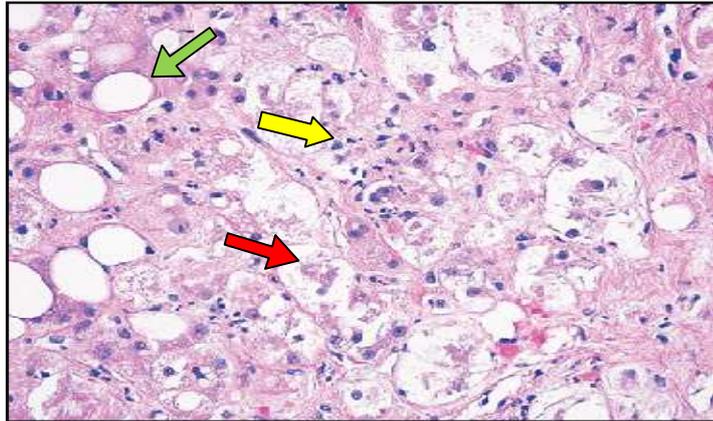


Figure 1. Histopathological features in a liver-biopsy specimen in AH.
Adapted from : - Michael R. Lucey M R, Mathurin P and Morgan T R .²⁶

II.1.6. Pathogenesis of liver injury induced by ethanol :

II.1.6.1. Mechanism of ethanol-induced hepatic steatosis

Macrovesicular steatosis occurs in all drinkers within a few weeks of drinking. A combination of increased lipogenesis and impaired fatty acid breakdown results in steatosis which worsens as the alcohol liver disease (ALD) progresses. In hepatocytes the most important pathway is oxidation of ethanol via alcohol dehydrogenase (ADH) to acetaldehyde. In mitochondria, acetaldehyde is converted by aldehyde dehydrogenase (ALDH) to acetate and in turn acetate is converted to acetyl coenzyme A (acetyl CoA) which leads the two-carbon molecule into the TCA (tricarboxylic acid cycle). This oxidation generates reducing equivalents, primarily reduced nicotinamide adenine dinucleotide (NAD), which contribute to reactive oxygen species production (ROS). The changes in the NADH–NAD⁺ potential in the liver inhibit both the oxidation of

triglycerides, fatty acids and the TCA and may thereby increase lipogenesis (Figure 2C1).^{26,27}

S-adenosylmethionine (SAME) is the primary methyl donor and a precursor to glutathione. Oxidative stress activation via long term alcohol consumption, through the activity of cytochrome P-450 2E1, that alters the intracellular balance between levels of S-adenosylmethionine (SAME) and S-adenosylhomocysteine (SAH), resulting in a decrease in hepatic methionine levels, decrease in the activity of methionine adenosyl transferase, the enzyme which converts methionine to SAME²⁸ and a decrease in the ratio of S-adenosylmethionine to S-adenosylhomocysteine. A decrease in these agents may contribute to alcohol induced liver injury, since S-adenosylhomocysteine exacerbates TNF- α hepatotoxicity, whereas S-adenosylmethionine diminishes it. Reduction in the enzymes that convert ((S-adenosylhomocysteine (SAH) to methionine , then convert methionine to S-adenosylmethionine (SAME))), lead to increase in homocysteine. This causes a decrease in glutathione synthesis which results in impaired clearance of oxidative species such as 4-hydroxynonenal (a marker of lipid peroxidation) and mitochondrial injury caused by a decrease in glutathione transport from the cytosol into the mitochondria. Increases in concentration of S-adenosylhomocysteine(SAH), stressing the endoplasmic reticulum (ER stress), that lead to release of sterol regulatory element-binding protein 1c (SREBP-1c) from ER stress (Figure 2C1).^{26,29}

Also ethanol increase in fatty acid synthesis by inhibiting AMP kinase (AMPK or 5' adenosine monophosphate activated protein kinase).²⁷ Inhibition of

AMP-activated protein kinase (AMPK) activates hepatic fatty acid synthesis by activating hepatic sterol regulatory element-binding protein (SREBP-1c) transcription.³⁰ These mechanisms lead to SREBP-1c initiating the transcription of genes involved in triglyceride and fatty-acid synthesis. Also ethanol has been shown to promote lipid metabolism by inhibiting peroxisome-proliferator-activated receptor α (PPAR- α). Decrease in the binding of peroxisome-proliferator-activated receptor α (PPAR- α) to DNA reduces the expression of genes involved in fatty acid oxidation (Figure 2C1).^{26,27}

II.1.6.2. Liver injury due to ethanol metabolites

Hepatocytes convert ethanol to acetaldehyde through three mechanisms: Alcohol dehydrogenase (ADH), cytochrome P-450 isoenzyme-1 (CYP2E1), and catalase.³¹ Lipid-peroxidation products can combine with acetaldehyde and with proteins to produce neoantigens, which can stimulate an autoimmune response. Against the background of steatosis and existing liver damage, heavy and continued drinking in some patients causes alcohol hepatitis. Two main mechanisms are involved: inflammation and oxidative stress.³²

II.1.6.2. a. Mechanism of ethanol-induced hepatic inflammation

Inflammation is a localized defensive response to tissue injury. Liver inflammation is the hallmark of alcoholic hepatitis. Ethanol ingestion increases gut permeability and facilitates translocation of LPS endotoxin from the small and large intestines to the portal vein and on to the liver (Figure 2A). Initiating events include expression of gut-derived lipopolysaccharides (LPS), interaction of LPS

with toll-like receptor (TLR4) receptors.³³ TLR4 adapter pathway activates NF- κ B in Kupffer cells, that is translocate to the nucleus in response to stress signals and binds to the promoter region of pro-inflammatory, thereby activating multiple cytokine genes.^{26,34,35} Kupffer cells are pivotal for the development of hepatic inflammation induced by ethanol.³⁶ The ongoing ethanol-induced LPS absorption may provide a continuing stimulus to Kupffer cells that perpetuates hepatic inflammation induced by ethanol(figure 2B).^{26,37}

Ethanol is broken down (i.e., metabolized) in the liver cells by two enzymes, alcohol dehydrogenase (ALD) and, particularly after chronic ethanol consumption, cytochrome P450 2E1 (CYP2E1). Both enzymes convert ethanol to acetaldehyde, a toxic substance. Some of the acetaldehyde interacts with proteins in the cells such as malondialdehyde, which results from lipid peroxidation, interact, through a covalent binding, with the reactive lysine residues of proteins, formation malondialdehyde-acetaldehyde proteins adducts (MAA) which called adducts, located on the membranes of hepatocytes. MAA proteins adducts capable to produce neoantigens, which can activate certain immune cells to produce various cytokines, including interleukins such as (IL-1, IL-2), interferon gamma (IFN- γ), and tumor necrosis factor alpha (TNF- α), that in turn attacks healthy liver cells, resulting in tissue damage (figure 2C2).^{26,38,39}

There are other sources of oxidative stress caused by ethanol to induce inflammation , that include granulocytes from the inflammatory process catalyzed by the enzymes NADPH oxidase and myeloperoxidase which cause generates highly reactive molecules known as reactive oxygen species (ROS), which

accumulate primarily in cell structures called mitochondria (figure 2B1).^{26,40} ROS normally are eliminated from the cells by compounds known as antioxidants, particularly a small molecule called glutathione (GSH). Alcohol, however, depletes the cell's GSH stores, thereby further exacerbating ROS accumulation in the mitochondria. This process leads to the release of cytochrome c from the mitochondria, which then activates enzymes called caspases and promotes production of IL-8 in the cell (figure 2C3).^{26,37} ROS can also activate ERK1/2, p38 MAPK kinases, and NF- κ B which stimulates TNF- α , complementing the LPS-induced pathways of TNF- α production and creating a vicious cycle of inflammation and oxidative stress (figure 2B1).^{26,41}

II.1.6.3. Mechanism of ethanol-induced Mallory bodies

In hepatocyte, ethanol is converted to acetaldehyde by the cytosolic enzyme alcohol dehydrogenase (ADH) and the microsomal enzyme cytochrome P-450 2E1 (CYP2E1). CYP2E1 may increase 5-20 fold in patients with alcoholic hepatitis, leading to increased electron leakage and release of ROS by CYP2E1 and mitochondria cause lipid peroxidation and produce protein carbonyls, that lead to inhibition of proteasomes.²⁷ Inhibition of the proteasome reduces the catabolism of damaged proteins and may contribute to the accumulation of an abnormal form of ubiquitin called Ub+1. Ubiquitin (Ub+1) is formed when the gene encoding ubiquitin is "misread" by the cell's machinery, resulting in synthesis of ubiquitin molecules that are longer than normal. When these abnormal ubiquitin-protein conjugates called cytokeratin filaments, they cannot be removed as easily as normal ubiquitin, thereby slowing down the degradation

of the attached protein, that lead to accumulation of cytokeratin filaments, and formation of Mallory bodies on liver histology. In addition, Ub+1 itself inhibits proteasome function and could thereby cause liver cell death (figure 2C4).^{26, 42}

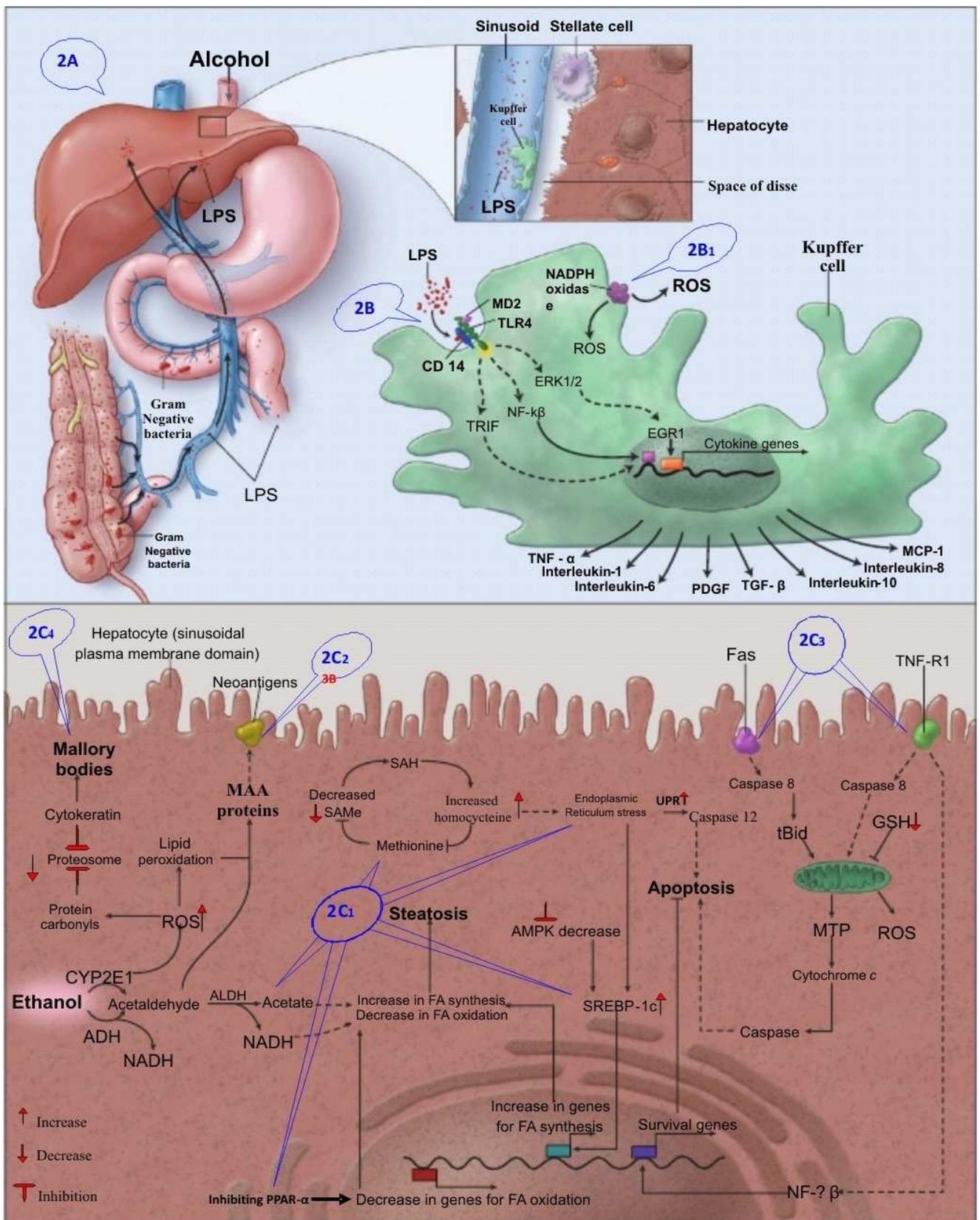


Figure 2. (A,B,C1,C2,C3,C4) Overview of the pathway of alcoholic injury resulting in the direct production of oxidative stress and the LPS-TLR pathway that culminates in production of cytokines and other inflammatory processes that result in hepatitis of the liver. Adapted from:- Michael R. Lucey M R, Mathurin P and Morgan T R.²⁶

II.2. NIGELLA SATIVA SEEDS

II.2.1. Description

Nigella sativa is bisexual and forms a fruit capsule which consists of many white trigonal seeds. Once the fruit capsule has matured, it opens up and the seeds contained within are exposed to the air, becoming black in color (black seeds). *Nigella sativa* and its black seed are known by other names, varying between places.⁴³ (fig. 3)



Figure 3 : Black Seed Flower , black Seed *Nigella* seeds: the miraculous Black Seed. Adapted from: <http://www.healthinfo21.com/2010/11/nigella-seeds-miraculous-black-seed.html>.⁴⁴

Nigella sativa Scientific classification:- Kingdom: Plantae, Division: Magnoliophyta, Class: Magnoliopsida, Order: Ranunculales, Family: Ranunculaceae, Genus: *Nigella*, Species, *Nigella sativa*. The scientific name is a derivative of Latin Niger (black). Original black cumin seed is *Carum bulbocastanum*. The botanical name is *Nigella sativa*, while In English, *Nigella sativa* seed is variously called fennel flower, nutmeg flower, Roman coriander, black seed or black caraway. It has Other names such as habbat al-barakah (Arabic), kaljeera (Assamese), kalo jira (Bengali), karum cheerakam (Tamil),

kalonji (Hindi/Urdu) or mangrail (Hindi), ketzakh (Hebrew), chernushka (Russian), çörek otu (Turkish), siyah daneh (Persian), karim jeerakam (Malayalam or in Sinhala).⁴⁵

II.2.2. The chemical composition of *Nigella sativa* seeds

The general chemical composition of the seeds is depicted in Table 2.

Table 2 : The general chemical composition of *Nigella sativa* seeds Constituent % Range (w/w). Adapted from: Ismail M Y M.⁴⁶

Constituent	% Range (w/w)
Oil	31-35.5
Protein	16-19.9
Carbohydrates	33-34
Fibre	4.5-6.5
Saponins	0.013
Moisture	5-7

II.2.3a. The chemical composition of *Nigella sativa* oil

The chemical analysis of *Nigella sativa* total oil revealed the presence of both a fixed oil and a volatile oil. The major component was the fixed oil whereas the volatile oil ranged from 0.4- 0.7% of the seeds' weight. The fixed oil chemical composition is outlined in Table 3.

Table 3 : The chemical composition of *Nigella sativa* fixed oil .(Adapted from : - Ismail M Y M.) .⁴⁶

Constituent	% Range (w/w)
Linoleic Acid	44.7-56
Oleic Acid	20.7-24.6
Linolenic Acid	0.6-1.8
Arachidic Acid	2-3
Palmitoleic Acid	3
Eicosadienoic Acid	2-2.5
Palmitic Acid	12-14.3
Stearic Acid	2.7-3
Myristic Acid	0.16
Sterols	0.5

Generally, there were no significant variations in the chemical composition of the fixed oils of seeds grown in Egypt, Sudan, Ethiopia, India, Turkey and Syria. However, Al-Jassir noted that the seeds grown in Qassim, Saudi Arabia, contained, in addition to the fatty acids depicted in Table 2, two more acids which were lignoceric acid about (1%) and myristoleic acid (0.18%) without the presence of eicosadienoic acid. Specific chemical analyses of the volatile oil started by Mahfouz and El-Dakhakhny and Canonica et al. These studies were complemented by most recent ones which revealed various pharmacologically active constituents that included Thymoquinone that may

attain up to 27.8% of the volatile oil (w/w), Carvacrol phenol which is also known as isothymol (5.8-11.6% (w/w)), p-cymene in the range of (15.5-31.7% (w/w)), α -pinene in the range of (9.3% (w/w)), α -terpineol in the range (2-6.6% (w/w)), longifolene (1-8% (w/w)), t-anethole (p-Propenyl anisole)benzene in range (0.25-2.3%(w/w)) and the reduction product of thymoquinonethymohydroquinone together with some esters about 16% .

II.2.3b. Non-oily components of *Nigella sativa* seeds

1. Minerals

Analysis of *Nigella sativa* seeds, ash revealed the presence of 0.5-1% calcium, 0.6% phosphorus, 0.6% potassium and 0.1% sodium of the total seeds weight .

2. Saponins

The major saponin in the defatted seeds of *Nigella sativa* is the glycoside α -hederin or Helixin or melanthin which on acid hydrolysis releases its sugar rhamnose / arabinose and gives the aglycone hederagenin (or melanthigenin) or caulosapogenin.

3. Alkaloids

Three types of alkaloids were isolated from the defatted seeds of *Nigella sativa*. These were identified as the indazole nigelicine, the isoquinoline nigellimine and its N-oxide and the indazole alkaloid nigellidine.⁴⁶

II.2.4. The therapeutic potential of *Nigella sativa* seeds

II.2.4.1. Anti inflammation of *Nigella sativa* seeds

Inflammation has been known to produce proinflammatory cytokines and diverse reactive oxygen species (ROS) and reactive nitrogen species (RNS) creating pre-disposition to various patho-physiological disorders.⁴⁷ The amelioration of the Experimental Allergic Encephalitis by TQ was examined, who showed potent effects, which were thought to occur via induction of glutathione.⁴⁸

TQ was found to decrease NF- κ B activation, thereby NF- κ B is a molecular target of TQ, where previous studies showed the effects of TQ on the inhibition of activation of NF- κ B in an experimental autoimmune encephalomyelitis in the rat model of multiple sclerosis. TQ has been shown inhibit of activation of NF- κ B in an experimental autoimmune encephalomyelitis. TQ was able to increase the red blood cell glutathione, and inhibit the activation of NF- κ B in the brain and spinal cord. These results clearly provide some early indication that many of the biological activities of TQ could in part be due to inactivation of NF- κ B and its downstream genes. Collectively, these results suggest that NF- κ B is a molecular target of Thymoquinone.^{49, 50, 51}

II.2.4.2. Antioxidant activity of *Nigella sativa* seeds

Both *Nigella sativa* oil and thymoquinone can partly protect gastric mucosa from acute alcohol-induced mucosal injury which is partly ascribed to their radical scavenging activity.^{52,53} Thymoquinone (TQ) was the main active constituent of the volatile oil of the black seed. It has been shown to exhibit

antioxidant property through different mechanisms in several recent reports. For example, it inhibits the production of 5-hydroxyeicosa- tetraenoic as well as 5-lipoxygenase products.⁵⁴ It has been shown to work as a scavenger of various reactive oxygen species including (ROS), superoxide radical anion and hydroxyl radicals. It has been shown that TQ could inhibit iron-dependent microsomal lipid peroxidation efficiently (MEOS).⁵⁵ The compound was observed to decrease cellular oxidative stress by inducing glutathione in experimental allergic encephalomyelitis.⁵⁶ Present findings suggest that Thymoquinone has a potent chemopreventive potential of inhibiting the process of carcinogenesis by modulating lipid peroxidation and cellular antioxidant milieu.⁵⁷ TQ have found react with GSH, NADH and NADPH chemically. Such reactions occurring under the physiological conditions clearly indicate the formation of two products, viz. glutathione dihydrothymoquinone after rapid reaction with GSH and dihydrothymoquinone (DHTQ) after slow reaction with NADH and NADPH, respectively. These compounds exhibit antioxidant capacity. These results indicate a possible intracellular non-enzymatic activation of TQ dependent upon GSH, NADH and NADPH are representing perhaps the 'cellular switch' for modulating cellular antioxidant defenses. The protective effects of Thymoquinone have been assessed, Results suggest that treatment with Thymoquinone increased GSH levels compared to normal levels and inhibited the in vitro production of superoxide radicals in enzymatic and non-enzymatic systems.⁵⁸ Active antioxidant components of black seeds of *Nigella sativa* plants have been shown capable of rendering protection against the development of methionine-induced

hyperhomocysteinemia (Hhcy) and its associated state of oxidative stress. Pre-treatment of rats with an oral dose of 100 mg/kg of TQ for 30 min and for one week provided complete protection against induced HHcy after methionine load (100 mg/kg). Treatment with the different doses of Thymoquinone (TQ) produced significant reductions in hepatic superoxide dismutase (SOD), and catalase (CAT) and glutathione peroxidase (GSH-Px) activities although neither produced any change in GST activity nor influenced reduced glutathione content in any of the tissues studied. These results revealed that Thymoquinone (TQ) and dihydrothymoquinone (DHTQ) acted not only as superoxide anion scavengers but as general free radical scavengers.⁵⁹

The effects of TQ on carbon tetrachloride (CCl₄)- induced hepatotoxicity have been investigated in male Swiss albino mice. Oral administration of TQ in a single dose (100 mg/Kg) resulted in a significant protection against the hepatotoxic effects of CCl₄. The result suggests that the protective action. The effects of TQ against CCl₄-induced hepatotoxicity may be mediated through the combined antioxidant properties of TQ and its metabolite DHTQ.^{60,50,51}

II.2.5. Side effects of *Nigella sativa* seeds

Nigella sativa seeds are a safe and effective herb that can be used by almost anyone. No irritations or side effects are caused when the right dose is correctly applied. Oil extracted from *Nigella sativa* seeds may cause kidney or liver damage when used in high doses. These severe side effects of treatment were observed in rats given 2 g/kg or more of *Nigella sativa* oil, according to Memorial Sloan-Kettering cancer center risk (MSKCC). In addition, pregnant women

should not take *Nigella sativa* seeds if their wombs are sensitive unless otherwise instructed by a medical professional (Many Muslim women take it while pregnant and no harm has been found).^{61, 62}

II.3. Animal models of ethanol-induced liver damage

Animals have been administered ethanol by various methods in attempts to try to develop liver lesions resembling those seen in human ALD. The animals continued to gain weight with ethanol concentrations up to 50%, showed increased ethanol metabolism, and sustained elevated blood ethanol levels, but liver damage occurred at most in only a minority of the animals. To overcome the failure of most animals otherwise to consume higher amounts of ethanol voluntarily, it has been administered in a nutritionally adequate liquid diet that provided a maximum of 35-40 % of total calories from alcohol. This situation resembles that of many alcoholics, who often receive more than 50 % of their total energy as ethanol. In rats this has been achieved by administration of ethanol as a component of the liquid diet, either orally or by forcing intragastric infusion.⁶³

II.3.1. Intragastric ethanol feeding model

In this model ethanol liquid diet is fed through intragastric catheter. As in the oral feeding model, controls are infused with isocaloric amounts of ethanol-free diet, with carbohydrates replacing ethanol. The gastrostomy tube is usually implanted on adult rats weighing 300-400g, but rats weighing 200-250g have also been used. In younger animals the liver injury was found to be more severe and

the fibrotic activity stronger. Either single or double gastrostomy cannulas have been inserted via the neck into the stomach. There is previous study that intragastric administration of ethanol as part of a low carbohydrate diet (or a high unsaturated fat diet with 42% to 49% of total energy as ethanol) to Wistar rats is also a key component required for the production of ethanol-induced steatosis with additional inflammation and necrosis, that also Inflammation and necrosis were significantly greater in the livers of rats fed intragastrically than orally. The intragastric feeding technique was recently applied to mice. One problem is that their metabolism of alcohol is so rapid (i.e. 25 g/kg/d) that it is difficult to feed them enough ethanol without compromising their nutritional balance. This may explain the frequent loss of animals reported during intragastric ethanol feeding to mice.⁶³